INFECT Report Summary

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Final Report Summary - INFECT (Improving Outcome of Necrotizing Fasciitis: Elucidation of Complex Host and Pathogen Signatures that Dictate Severity of Tissue Infection)

Executive Summary:
The overall goal of INFECT is to advance our understanding of the pathophysiological mechanisms, prognosis, and diagnosis of the multifactorial highly lethal necrotizing soft tissue infections (NSTIs). NSTI’s are rapidly spreading infections that may cause extensive soft tissue or limb loss, multiorgan failure and are associated with a considerable fatality rate. It is undisputed that rapid diagnosis and prompt intervention is directly related to survival. The initial presentation may be limited to unspecific symptoms such as tenderness, swelling, erythema and pain. Thus, diagnosis and management are difficult due to heterogeneity in clinical presentation, in co-morbidities and in microbiological aetiology. There is an urgent need for novel diagnostic and therapeutic strategies in order to improve outcome of NSTIs. To achieve this, a comprehensive and integrated knowledge of diagnostic features, causative microbial agent, treatment strategies, and pathogenic mechanisms (host and bacterial disease traits and their underlying interaction network) is required.

INFECT was designed to obtain such insight through an integrated systems biology approach in patients (WP2) and different clinically relevant experimental models (WP1 and WP6). The work flow includes a comprehensive set of analyses (WP3 and WP5) followed by integration of results in advanced computational platforms, which enabled generation of pathophysiological models of the disease (WP4) and advanced understanding of the underlying mechanisms and hosts-pathogen interactions. The results were translated into novel diagnostic tests (WP7) and improved patient management (WP2 & 8). The work was conducted by the INFECT consortium, which consisted of a team of multidisciplinary researchers, clinicians, SMEs and a patient organization, each with a unique expertise, technical platform and/or model systems that together provided the means to successfully conduct the multifaceted research proposed and efficiently disseminate/exploit the knowledge obtained (See figure 1, appendix 1).

Key achievements of INFECT include:
- Establishment of the world’s largest NSTI patient cohort with extended clinical registry and associated biobank providing a unique resource for the proposed studies.
- Advanced insight into the clinical aspects of NSTIs providing the basis for evidence-based guidelines for patient management and care.
- The systems medicine analyses within INFECT have substantially advanced our understanding of these life-threatening infections, including the identification of novel pathogenic mechanisms and specific host and bacterial disease traits associated with disease outcome.
- The results demonstrate that the pathophysiology of NSTI are influenced both by the causative microbe and by host factors, underscoring the need for patient stratification and implementation of tailored therapy/personalized medicine in these infections.
- Multiplex diagnostic tools for rapid pathogen identification and monitoring of disease associated biomarkers have been developed and tested in the clinical setting.
- The novel understanding of the disease mechanisms of these infections has resulted in changed clinical practice related to antibiotic usage as well as use of immunomodulatory treatments.
- Fostering the new generation of clinical and preclinical scientists within the field of systems medicine in infectious diseases.

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Research and Innovation
Overall, INFECT has proven the value of systems medicine approaches in acute infectious diseases to achieve improved diagnostics and therapeutics to improve patient disease outcome.

Project Context and Objectives:
The tasks of INFECT were undertaken in 8 highly integrated WPs; all designed to jointly address the specific objectives of the project.

A central aspect of the INFECT project is the prospective enrolment of patients with NSTI and collection of biobank samples as well as the causative microbes (WP2, clinical partners 2, 3, 4, 5, 6). The clinical partners succeeded in enrolling 409 NSTI patients with completed clinical registries (>2000 variables), and collecting associated biobank samples (> 6000). This represents the world’s largest NSTI patient cohort and is a unique resource for the project.

Results obtained related to objective 1 “Unravel specific mechanisms underlying diseases signatures though a bottom-up systems approach applied to clinically relevant experimental settings”.

The major tasks here involved establishment of experimental models optimized for NSTI infection, including a model-driven, forward genetics approach using advanced murine models (ARI BXD and HLA class II transgenic mice) (WP1 headed by partner 15), as well as a human 3D artificial tissue model system (WP6 headed by partner 1). The models were successfully established and proven to be robust tools for modelling NSTI. Some key findings obtained through the use of these models were:

A systems genetics approach using S. pyogenes infected ARI BXD mice identified genetic loci and gene networks that strongly predicted critical disease phenotypes, i.e. survival, weight loss and lesion size in NSTI (WP1, partner 15). The IL1B network was identified as a key regulator of severity of NSTI. This finding was confirmed by studies using the same S. pyogenes strain in infections of the human tissue model (WP6, partner 1) as well as by analyses of patient tissue biopsies (WP5, partner 1).

A reductionist approach using humanized transgenic mice expressing HLA-Class II genes showed that variations in HLA-II alleles determine severity of NSTI pathogenesis, specifically mice expressing DR3 showed larger lesions and high mortality to NSTI with S. pyogenes isolate 5448 and to a lesser extent to the INFECT isolate 2006. Hence, revealing both host genetic factors influencing severity of NSTI but also differences based on the infecting isolate (WP1, partner 15).

Using Ingenuity Pathway Analysis Tools, partner 15 identified PPARγ as one among the key upstream regulators that drive differential responses whose expression was significantly downregulated during NSTI. Data also revealed that S. pyogenes disseminated into adipose tissue and impaired adipogenesis. Based on these findings, novel intervention strategies have been tested (see obj. 4) and also customization of the human tissue model to include adipocytes (WP6, partner 1).

Results obtained related to objectives 2 and 3 “Apply a top-down systems biology approach to NSTI patient samples to pinpoint key host and pathogen factors involved in the onset and development of infection”, and “Identify and quantify disease signatures and underlying networks that contribute to disease outcome”.

These two objectives are based on the utilization of the prospective NSTI patient cohort with associated clinical data and linked biobank samples and the causative bacterial isolate(s) (WP2, partners 2, 3, 4, 5, 6), as well as the experimental models (WP1, partner 15, and WP6, partner 1). The successful collection of the large INFECT cohort and biobank enabled a systems medicine statistical approach (WP3 and WP4; partners 8, 9, 10, 11, 14). System-wide analyses (genomics, transcriptomics, proteomics, metabolomics) of both pathogen and infected subjects/models (WP1, WP3, partners 1, 8, 9, 10, 14, 15) have been undertaken and analysed through solid multivariate statistics and pathway analyses (WP4, partners 8, 9, 10, 11) to delineate factors/pathways/biomarker sets that contribute to disease severity and outcome. The multiple and heterogeneous data sets from WP1-3, 5 and 6 were aggregated in a dynamic, relational database (WP4, partner 9, 11). Some main achievements/findings of this work were:

On the pathogen side, typing methods and a comprehensive genome database were established for S. pyogenes, S. dysgalactiae and S. aureus NSTI isolates; all prevalent causes of NSTI (WP3, partner 8, 10). Statistical analyses of microbiological characteristics and clinical variables revealed a striking link between site of infection and microbial aetiology, e.g. NSTI of the upper or lower extremities was associated with monomicrobial S. pyogenes while NSTI located to the abdomen/ano-genital area was associated with polymicrobial infection (WP2, WP3, WP4; partners 2, 3, 4, 5, 6, 8, 11).

The frequent occurrence of multi-species NSTI cases spurred the first comprehensive culture-independent characterization of the NSTI pathobiome, providing insights into the microbial community network and analyses of species distributions/
interactions (WP3, partner 8).

The microbial community profiling was integrated with analysis of host-microbe interactions by RNA-sequencing, which identified differences in the pathophysiology of monomicrobial streptococcal and polymicrobial NSTI (WP3, WP4, partners 8, 9). While pathogenic streptococci express a wide range of virulence factors that mediate the different steps of infection, the pathogenicity of polymicrobial NSTIs is dependent on the co-occurrence of multiple bacterial taxa, which complement each other to enhance the virulence of the bacterial community as a whole. These differences result in distinct patterns of molecular host aetiology-dependent molecular pathophysiology.

Pathway analyses of RNAseq data of S. pyogenes NSTI patient biopsies in comparison to healthy controls, were done with a specific focus on the immune responses (WP3, WP4, partner 1, 8, 9, 11). Several over-represented pathways were identified in the NSTI patients, including neutrophil degranulation (see below result point), and specific signaling pathways. Furthermore, RNAseq data from S. pyogenes infected ARI BXD mice identified the same set of implicated pathways, and their upregulation was linked to severity of NSTI (WP1, partner 15). Similarly, using the human skin tissue model with embedded monocytes revealed an upregulation of the implicated signaling pathways (WP6, partner 1). These typical polarizing signals were confirmed in S. pyogenes infected patient tissue biopsies by use of multiparameter confocal microscopy analyses (WP5, partner 1). The data further implied that the responses are pathogen-specific with different patterns for S. aureus and S. pyogenes. The differential response by S. aureus is in line with its preferential adherence and invasion of particular cell types, where the bacteria also causes substantial cell death that likely is an important contributor to the tissue pathology of S. aureus NSTI (WP3, partner 10).

The RNAseq data implicating neutrophil degranulation as a disease trait verifies the in vitro studies implicating neutrophil degranulation as a key pathogenic mechanisms contributing to S. pyogenes NSTI (WP3, WP5, WP6, partner 1).

Patient tissue biopsies were analysed to verify identified pathogen and host traits (WP5, partner 1). One key finding of these analyses were the demonstration of biofilm formation in over 30% of S. pyogenes NSTI patients. This has great implications for the antibiotic efficacy and hence choice of antibiotics.

Also systemic host responses were assessed through analyses of plasma samples using metabolomics and customized multiplex protein assays including panels of inflammatory and metabolic factors (WP3, WP4: partner 1, 2, 6, 9, 11). The results of these analyses confirms the dysregulated host response in NSTI and importantly reveals that it is not limited to the tissue site but is also reflected systemically, which is of importance for the diagnostic tool development (WP7, partner 16).

A death prediction model was developed implementing machine learning approaches and using selected “early” clinical variables, like baseline, demographic and early measurements (WP2, WP4: partner 9, 11, 14). From those, a set of best predictor variables were selected using a Random Forest algorithm. Both Random Forest and Support Vector Machines were deployed as machine learning tools for the actual prediction. The prediction model was included as a feature for a prototype mobile app, which allows users/clinicians to input the values for a small set of predictive early parameters in order to receive a probability of patient death/amputation after a certain time period post diagnosis, e.g. after 30 days. In another approach, the set of clinical variables was combined with gene expression data from the RNA-Seq experiments, though with less success than using only clinical parameters.

Through group-wise principal components analysis of the plasma metabolomics data, key metabolites significantly altered in the NSTI patients as compared to uninfected controls were identified (WP3, WP4, partners 1, 9, 14). These metabolites have then tested in in vitro biofilm assays, and some found to significantly affect bacterial growth and biofilm formation (WP3, partner 1). Based on these findings a 3D cellular automata model was developed to dissect the conditions needed for the formation of biofilm of bacteria when in contact with human tissue (WP4, partner 14). The 3D cellular automata model is a computational model that simulates the interactions between bacteria in a spatial context, and represents the bacteria using their genome scale metabolic models, as well as taking into account gradients of stress signals, nutrients, and diffusion of metabolites in 3D.

In conclusion the results related to objectives 1-3 provides evidence that the pathogenic mechanisms of NSTI vary depending on microbial aetiology and co-morbidity, and involves distinct dysregulated host immune responses requiring a tailored immunotherapeutic approach in the individual patient. Hence, providing a strong foundation for future work towards personalized medicine in NSTI and in other severe infectious diseases associated with a dysregulated immune response such as sepsis.
Results obtained related to objective 4: “Identify novel therapeutic strategies for NSTI”.

A key aim of INFECT has been to improve therapies for NSTI driven by the fact that these infections are associated with significant risk for loss of lives and limbs, even in young previously healthy individuals. This was tackled in two ways: (i) two novel therapeutic strategies (intravenous immunoglobulin; IVIG and hyperbaric oxygen treatment; HBO) were evaluated for clinical efficacy and/or mechanistic action, and (ii) through identification of novel targets revealed by the integrated systems biology approach in WP1-WP6. Some key findings include:

- Analyses of patient plasma pre- and post-IVIG therapy revealed that the treatment resulted in inhibition of streptococcal virulence factors (WP3, partner 8). In addition, a randomized clinical trial of IVIG-therapy versus placebo in NSTI was conducted by partner 2 (WP2). The results showed that IVIG therapy had no beneficial effect in NSTI patients of all aetiologies. However, the data indicated that specific subgroups of patients, i.e. S. pyogenes NSTI, may benefit from the therapy.
- A detailed review of HBO therapy are being finalized to elucidate how HBO-therapy has been applied (target patient population, timing, dosages) at the different INFECT clinical sites. The results will be useful to predict which patients benefit the most, which will guide patient stratification and future clinical trials.

Results related to objective 5 “Exploit identified disease traits for the innovation of optimized diagnostic tools”.

This objective aimed to exploit the results obtained in WP1-6 to design, develop and validate a multiplex diagnostic tool (WP7) suitable for the clinical needs associated with care of NSTI patients, i.e. early and rapid identification of pathogens and host immune and organ status. To achieve this, a diagnostic SME (partner 16) employed two strategies; one applying compact sequencing for pathogen detection and one compact profiling for host responses. Key achievements included:

- Based on Anagnostics’ hybcell technology (partner 12), two prototype tests were developed by Anagnostics (partner 12) and further developed/redesigned by Cube (partner 16).
- A clinical on-site validation of the diagnostic tool for host responses (partner 16) was conducted by partner 2 (WP7). This analyses focused on plasma samples from the NSTI patients revealing that some, but not all markers, showed satisfactory correlation with comparative lab results. A combination of known and new markers were found to be predictive for key clinical outcomes, i.e. acute kidney failure and 30-day mortality at a level equal to SOFA scores, and better than conventional biomarkers CRP and PCT. The operation of the hybcell technology for plasma samples was found to be easy, fast and reliable with a relatively short staff training period. With further refinement, the system may be useful as a frontline diagnostic tool to improve timing and precision of the NSTI diagnosis and interventions.
- To further improve the ease of use, the test was validated for whole blood as well (partner 16).

- Combinations of biomarkers (two biomarkers, combined with help of logistic regressions) have been examined to stratify acute kidney failure (AKI) and mortality. The combination of Myoglobin and Cystatin C supersedes the prognostic capability of any single marker (with an AUC of 0.86). The prognosis of mortality could not be improved by any combination. The high multiplex capability and the outlook to elaborate superior biomarker combinations in addition to the bedside utilization represent advancement in diagnostics and potential for future clinical use.

Results related to objective 6 “Translate the advanced knowledge generated in INFECT into evidence-based guidelines for classification and management of NSTI”.

The comprehensive insight of clinical, therapeutic and pathogenic aspects of NSTI provides the foundation for evidence-based guideline for classification and management in NSTI (WP2, partners 2, 3, 4, 5, 6). Nonetheless, clinical guidelines must be developed by an independent advisory group. The INFECT consortium has therefore established an intentional agreement with the The Scandinavian Society of Anaesthesiology and Intensive Care Medicine (SSAI).
A number of dissemination activities have been undertaken, including:

The patient organization (partner 13) together with the NSTI clinical partners (2, 3, 4, 5, 6) has ensured an efficient dissemination of INFECT’s advances to the clinical community as well as to policy makers and the society in large, not the least to patients and their relatives.

- A contact with SSAI has been established for the preparation of clinical guidelines (WP2). Also through this collaboration, a 1-day postgraduate training workshop will be held in association with the international SSAI meeting 2019 (WP8).

Other key dissemination activities include:

- A book volume on NSTI will be published by Springer Nature. The target groups are health care professionals, scientific community and medical students/residents (WP8, all partners). This volume is due in 2019.
- Short videos, including also patients and the patient organization (partner 13), targeting the society at large is being finalized and will be published on Youtube (September 2018).
- 53 publications in well renowned journals has been published.
- Key contributions at international meetings, such as the Lancefield International symposium on Streptococcal infections and infective diseases, European conference of Clinical microbiology and infectious diseases, European Association Systems Medicine Conference, and Nordic Society of Clinical Microbiol and Infectious Diseases conference.

Project Results:

4.1.3.1 WP1 Systems genetics approach in a murine model of NSTIs

The objectives of WP1 were to through the use of an experimental murine model of NSTI:

- Map quantitative trait loci (QTL) harbouring genes with a high statistical likelihood of modulating susceptibility/outcomes of NSTI,
- Determine host susceptibility/outcome in relation to specific pathogens that cause NSTIs,
- Determine how variations in both the bacteria and in the host genetic content alters the pathogenic strategies and/or the host defense mechanisms, and
- Test clinical efficacy of novel adjunctive therapies.

The results achieved in this WP are described below and relates to the WP specific Tasks:

Task 1.1 Apply forward systems genetics approach to map QTLs harbouring genes with a high likelihood to be involved in modulating susceptibility/outcomes in an NSTI model.

Partner 15 established an NSTI BXD mouse model and using unbiased systems genetics approach, partner 15 identified genetic loci and gene networks, that strongly predicted survival, weight loss and lesion size in NSTI. Partner 15 analysed disease phenotypes in the context of BXD genotypes and identified highly significant QTLs on Chromosomes 2 and 7 that strongly predicts NSTI survival and weight loss respectively. Further Partner 15 identified suggestive QTLs on chromosomes 6 and 8 for lesion size. In search of key regulators that modulate NSTI susceptibility, Partner 15 demonstrated that: a) Host and gender are important regulators of GAS NSTI, b) D2 host were most susceptible than B6 host, and female mice of D2 and other strains of BxD were more resistant than male mice, c) Age and body weight were additional host factors that were significant predictors of survival, d) Host genetics influences GAS burden, bacteraemia and dissemination, and e) forward systems genetics approach revealed IL-1b was the key proinflammatory mediator of susceptibility to GAS NSTI. This work was summarized in the paper of ChellaKrishnan et al. PLoS Pathog 2016.

Task 1.2 Determine the host susceptibility/outcomes in infection in relation to specific pathogens that cause NSTI associated with various co-morbidities.

In determining how variations in bacteria can alter the bacterial pathogenic strategy (WP3, partner 1, 10, 15), Partner 15 validated using their murine model (WP1) of Staphylococcal infections how a single point mutation in phenotypic variants of ST22 MRSA strains can significantly alter virulence properties (Maipady Shambat, et al. Scientific Reports 2016). Partner 15 made a significant breakthrough in advancing knowledge of NSTI pathogenesis. Transcriptome analysis of skin
samples from mice infected with GAS revealed previously unknown niches for GAS adaptations in the host during NSTI that might contribute to NSTI pathogenesis.

Task 1.3 Determine how variations in both the bacteria and the host genetic content, alter the bacterial pathogenic strategy and/or the host defence mechanisms.

In determining how variations in both bacteria and the host genetic content alter bacterial pathogenic strategy, studies in WP1 (partner 15) demonstrated:

1. Host responses to GAS isolates with varying virulence were determined on D2 mice most susceptible to NSTI. SpeB activity was determined on representative GAS isolates selected based on their emm type.
2. There were survival differences in D2 mice, 2 out of 6 D2 mice infected with GAS 2002 (INFEKT clinical isolate-M12 type) and 1 out of 4 of the D2 mice infected with GAS 8003 (M3 type, reference strain) were dead by 48 hours post infection.

3. D2 mice infected with GAS 8003 showed lower CFU compared to infections with other GAS isolates. This difference was statistically significant when compared with GAS 2006 and GAS 5448. Levels of IL-1β transcripts in the skin at the site of infection were comparable in response to each of the different GAS isolates. However, at 72h post infection, plasma levels of IL-1β was significantly higher in infection with M1-5448 isolate compared to infections with INFEKT isolates 6026 and 6033 (M4 and M63 types respectively) (P<0.05).

4. Differential gene expression of upstream regulatory genes that drive the production of IL-1β was investigated by qRT-PCR in infected skin of D2 mice infected with GAS isolates with varying virulence. Not surprisingly, all the GAS isolates with varying virulence effectively induced inflammasome related genes and IL-1β, however, caspase 1 whose expression and activation are associated with IL-1β and IL-18 release was significantly downregulated. IL-18 expression mirrored Caspase-1 expression such that IL-18 was also significantly downregulated.

5. The expression changes in a few GAS genes (M protein, Mga, SpeB, CovR, CovS, SmeZ, HasA and RopA, gyrase, DNAseB) upon in vivo infections in D2 host were investigated by Partner 15. Consistently, GAS M protein and Mga showed high levels of expression in the host compared to other GAS genes.

6. In studying how host HLA-II allelic variations modulate susceptibility, Partner 15 demonstrated distinct polarization into Th1/Treg subsets in their HLA-II transgenic mice models. These findings lay the foundation for an as yet unidentified role for HLA-II alleles in regulating Th1/Treg stability during NSTI.

Task 1.4 Determine the efficacy of novel therapeutic interventions in the NSTI murine model.

Partner 15 undertook several therapeutic interventions in the BxD and HLA-II transgenic murine models of NSTI, including IvIg, Ciprofloxacin, and Clindamycin in conjunction with immunomodulators. Partner 15 evaluated the effect of the interventions in NSTI susceptible mice and measured their ability to reduce bacterial burden and dissemination, wound healing, lesion area and levels of pro and anti-inflammatory mediators (Manuscripts under preparation).

4.1.3.2 WP2 Clinical registry of NSTIs and associated isolate and biobank collection

The Scandinavian study group of NSTs (partners 2-6) will establish a prospective study of NSTI patients at major referral centers in Denmark, Sweden and Norway to obtain clinical samples that will be linked to detailed clinical information. The specific objectives were to:
- Establish a joint Scandinavian clinical registry
- Map quantitative trait loci (QTL) harbouring genes with a high statistical likelihood of modulating susceptibility/outcomes of NSTI,
- Prospectively enrol NSTI patients
- Collect clinical isolates and patient samples for the centralized biobank
- Generate evidence-based guidelines for classification and management of NSTIs
The results achieved in this WP are described below and relates to the WP specific Tasks:

During 2013-June 2017, a total of 525 patients were registered and screened within the INFECT cohort. In collaboration with partners in the WP2 group (here especially partner 2 (RH) and partner 6 (UiB) and partners 9 and 11, the database has been cleaned for data entry mistakes, missing data recovered. By re-evaluating and double checking data, uniformity in securing the proper registration of infectious agents (microbiology) in each and every patient has been performed. Cases of patient enrolments where diagnose was doubtful have been re-evaluated by the WP2 group and joint conference decisions made on their recruitment status.

The INFECT registry (electronic case report form) contains clinical data on all the patients, from debut of symptoms, number of surgical interventions- and descriptions, blood samples, treatment modalities, microbiology, length of stay and death (figure 2.1; appendix). In total, more than 2000 variables exist for each patient.

Final reporting and official closure of database:

The WP2 group (partners 2-6) have secured and exported all of the 409 clean patient data sets to the INFECT consortium to be worked with in corporation with partners 1, 7-11 and 14.

• Task 2.1b. Corporation with INFECT partners on clinical data analysis.

During 2017/18 the WP2 group team leader have hosted 4 international meetings with the relevant partners 2-6 and 9 and 11 as well as the INFECT consortium. A statistical- and clinical data analysis plan for the main clinical scientific reports has been created with partners 9 and 11 and published. Furthermore, the WP2 group have been cooperating with partners 9, 10, 11 and 14 using clean dataset of the 409 prospectively enroled patients.

Important achievements task 2.1 during this reporting period:
1. All 409 patient data sets have been delivered and the database has proven its operational capacity and feasibility.
2. The clinical data base has been re-verified, checked and declared as clean from data entry mistakes with final closure on the 1st of June 2018.
3. The first scientific reports are emerging – see publication list below.

Task 2.2 prospectively enrol NSTIs patients according to the estimated rate of patients to the different clinical centres. In tabular form the inclusions of patients into the INFECT cohort were distributed as given in figure 2.2 (appendix).

Important achievements task 2.2:

1. To sum up – we achieved our main target of including more than 400 patients during the 4½ year inclusion period. The rate of inclusion was stable throughout the study period.
2. This is the world’s largest prospectively enrolled patient cohort from the INFECT multicentre study with 409 patients included, fully monitored with clinical data, microbiome, blood- and tissue samples and one year survival follow-up.

Task 2.3 establish local teams to collect clinical isolates and patient samples for the centralized bio bank on a 24/7/365 time basis for all patients included into the INFECT study.

• Task 2.3 a SOP’s for tissue and blood sampling from patients with NSTI (Figure 2.3 appendix):
The WP2 group have jointly worked together with partners 1 and 8 during 2017 where all clinical partners have contributed. Shipping of Biobank samples have been continuously processed to partner 1 and 8 throughout 2017. Additional control blood samples have been collected during 2017/2018.
Important achievements task 2.3 during the study period:
1. A biobank has been established with 409 individual patient samples from well-defined, prospectively enrolled NSTI patients.
2. We have collected approximately 6000 samples for the entire INFECT biobank.
3. Scientific reports in collaboration between partners 2-6, 1, 7 and 8 have been published.
4. Enrollment of INFECT controls: Each center contributed with 5 healthy controls – these have been collected at WP2 partners 3, 4 and 6. At Rigshospitalet in CPH (partner 2) we extended the ethical permits for 2018 including the formal use of the following patients included into the INFECT cohort, now as controls:
   - Project BIONEC collected control samples (65 surgical controls consisting of patients undergoing elective orthopedic operations, i.e. in non-infected, non-septic patients where the surgical trauma effect on biomarkers from the human innate immune response can be isolated, described and quantified in comparison to NSTI patients)
   - The project ENDOPAT collected control samples (20 diabetics + 20 non-diabetics) with similar comorbidities as NSTI patients and receiving elective, hyperbaric oxygen therapy without having sepsis and septic-shock.
   - An additional 10 HBO patients with comparable comorbidities in non-NSTI patients without sepsis and septic-shock.
   - An additional 11 Healthy controls (3 day sampling as for INFECT NSTI patients).
   - An additional 14 patients with cellulitis were entered as controls from partner 8 UiB.
All together we have 155 individuals available as controls with SOP of sampling equal to the INFECT procedures.

Task 2.4 Generation of evidence-based guidelines for classification and management of NSTI’s Developing new clinical guidelines on the management of NSTI patients.

Our work has identified several important markers for predicting patient outcome that may now form the basis for modelling and machine learning to improve bedside clinical decision making. In collaboration with partner 11 a random forest analysis on predictors for outcome have so far identified baseline INR, lactate, Noradrenalin max infusion, first SOFA score and URINE output to be good predictors. From the data analysis published to this date we have found the following: complement-pathway activation in NSTI implicating only baseline Ficolin-2 was associated with short- and long-term mortality (Hansen et al J Innate Immun 2016); markers of inflammation in NSTI include pentraxin-3 (PTX3) (Hansen et al Crit Care 2016); RH IL-1β and IL-10 had the strongest association with 30-day mortality (Hansen et al Sci Rep 2017). In addition, RH partner 2 conducted a clinical trial on the use of IVIG therapy in NSTI (Bruun Madsen, et al Int Care Med 2017) showing that IVIG had no benefit in NSTI of all aetiologies, but potentially in NSTI subgroups, such as those caused by streptococci.

Accordingly, this work will continue using the entire INFECT cohort of 409 patients giving more precise and better predictive tools at hand. Furthermore, these data will be integrated in modelling and results be part of management guidelines. In corporation with the Scandinavian Society of Anesthesia and Intensive Care – SSAI - and their international, scientific meeting in Copenhagen 2019 the INFECT consortium will organize a workshop on the subject NSTI – pathophysiology, characteristics and treatment. Now and future directions.

The workshop will be available as part of the pre-congress meetings which will be advocated on the SSAI congress website. The location of the workshop is at the University Hospital in Copenhagen Rigshospitalet on the 27th of August 2019. Organizer is partner 2 (RH) from the INFECT consortium in corporation with partner 6. The INFECT/NSTI workshop at the SSAI meeting will include:

- Introduction to NSTI infections
- Pathogenesis
- Diagnosis, clinical management
- The return to life – patient’s perspectives
- Future aspects: Systems medicine approach to improve patient management in NSTI
- Guidelines and recommendations in NSTI -development, updates
The INFECT consortium will be hosting a workshop on the below scheduled meeting (figure 2.4; appendix) as a pre-congress event on the treatment of NSTI patients. Congress participants will be able to register for this workshop through the SSAI website during this autumn 2018. This work will subsequently pave the way for a joint effort in creating an SSAI based working group – holding significant number of members from the INFECT consortium - new treatment guideline based on the data generated by the INFECT consortium.

4.1.3.3 WP3 Identification of host and pathogen traits affecting disease outcome

The main objective of this work package was to identify specific pathogen and host disease signatures. Specific objectives are to apply systems biology approaches to:

- Identify pathogen traits/pathways associated with disease outcome
- Perform comparative virulence and expression profiling of clinical NSTI isolates
- Explore tissue-specific properties of isolates from blood and tissue of the same patient
- Apply transcriptomics, proteomics and metabolomics to selected sets of host & pathogen samples
- Identify host traits/pathways that contributes to tissue pathology and/or systemic toxicity

A summary of key findings achieved in this WP are described below in relation to WP specific Tasks:

Task 3.1 Identifying pathogen signatures associated with disease outcome.

From the patient cohort (WP2), 143 streptococcal isolates were obtained comprising 85 distinct strains originating from 42 mono- and 38 polymicrobial infections. The epidemiologic typing confirmed not only the dominant role of S. pyogenes but also the contribution of S. dysgalactiae ssp. equisimilis and of opportunistic streptococcal pathogens. Among S. pyogenes isolates the serotype M1 was dominating, but also the serotypes M3, M28 and M87 were isolated in considerable high numbers. S. pyogenes is able to secrete an arsenal of highly potent exotoxins. To evaluate their role during the acute phase of infection partner 8 used a multiplex PCR to rapidly detect 21 streptococcal exotoxin genes. Analysis revealed that speG was present in the genomes of all analyzed S. pyogenes isolates. A minimum of 3 superantigens were present per strain. In contrast to the high prevalence of NSTIs caused by streptococci, only five cases were clearly caused by S. aureus. An analysis of 27 strains, including NSTIs, necrotizing cellulitis, myositis and cellulitis, caused by S. aureus from the French National Reference Center by a DNA microarray, covering 185 distinct genes did not reveal the presence virulence factors specific for NSTI isolates (partner 10). However, the genes encoding the cytotoxin PVL were strongly linked to primary skin infections as compared to colonization isolates.

To characterize the NSTI pathobiome, partner 8 applied 16S ribosomal DNA profiling to tissue biopsies of 148 patients. Monomicrobial infections were primarily caused by S. pyogenes. S. dysgalactiae and S. agalactiae, pathogens rarely reported as associated with NSTIs, were frequently found, whereas Proteobacteria were rarely observed and only five cases of S. aureus infections were identified. Also Clostridia sensu stricto, historically considered important causes for clostridial myonecrosis, were only seldomly observed. Polymicrobial NSTIs were associated with varying bacterial communities, however they were typically composed of the Clostridiales genera Parvimonas and Peptostreptococcus, the Bacteroidiales genera Prevotella, Porphyromonas and Bacteroides as well as Fusobacterium spp. Phyotypes could usually be identified down to the species level and several of these are common members of the healthy human microbiota. Their contribution to the overall disease etiology is still underestimated as they are difficult to detect by culture-based methods. Consistent with clinical reports, the bacterial diversity of the pathobiome associated with NSTIs of the extremities was significantly lower than of infections localized at the head/neck or anogenital region, indicating a higher frequency of polymicrobial infections in local proximity to the body’s orifices. Accordingly, higher abundances of Bacteroides spp. were observed in anogenital infections than at those of the extremities, while Prevotella/ Porphyromonas/Fusobacterium exhibited increased abundances in infections of the head/neck area, highlighting the association between natural niches and contribution to NSTI pathophysiology for human pathobionts. To characterize microbial interactions within the NSTI pathobiome, partners 8 and 9 inferred bacterial co-occurrence patterns via network analysis (WP3, WP4) and observed significant negative associations between pathogens causing monomicrobial NSTIs and the diverse genera observed in polymicrobial infections. This indicates competitive exclusion between human pathobionts and pathogens. Using divisive clustering of co-occurring genera, highly interconnected clusters of bacterial genera predominately associated with polymicrobial NSTIs were observed, indicating that synergism is essential for the establishment and progression of polymicrobial necrotic tissue infections.
Task 3.2 Comparative whole genome analysis to identify pathogen- and tissue-specific traits.

To identify bacterial factors associated with the development of NSTI, a comparative whole-genome sequencing approach was employed. During the course of the project, 66 bacterial isolates comprising 35 streptococcal NSTI-associated strains (26 S. pyogenes, 6 S. dysgalactiae and one S. anginosus, S. constellatus and S. oralis, respectively) and 31 S. aureus isolates (including 17 NSTI isolates) were sequenced (Partners 8, 10). In accordance with the serological typing, in silico typing and multi locus sequence typing of novel NSTI causing S. pyogenes isolates highlighted the dominance of the M1 serotype and ST28 in NSTI infections. These types are also the most frequent in northern Europe and in databases. A comparison of virulence factor profiles of NSTI causing isolates and NCBI reference genomes, revealed no obvious differences. A core of potent virulence factors, like, exotoxin speB, and anti-proteolytic factor grab, is present in most genomes. Accessory virulence factors like certain adhesins and exotoxins are only present in some of the novel and reference S. pyogenes genomes. No specific virulence patterns or other genomic features were observed in NSTI causing isolates. A detailed comparison of S. pyogenes M1 strains was then performed to identify genomic features causing substantial differences in virulence and pathology. Only minor genomic differences were observed and analysis indicated that the progression from locally restricted to a systemic dissemination and hypervirulence seemed to be caused by multiple copies of prophage-encoded virulence factors and a dysregulation of virulence gene expression by covRS and rofA mutations.

A comparative genome analysis of 28 S. aureus isolates from NSTI and bacteremia revealed a high genomic variability, but no apparent discrimination between both invasive strain types (partners 8, 10). Phylogenetic analysis based on the core genome confirmed that NSTI and hematogenous strains were phylogenetically related. Further genomic comparison could not identify discriminant markers between the two groups. Due to the high number of variable genomic loci and the limited number of available genomes, distinct genomic features could not be identified. A link between distinct genomic features and the development of NSTI or bacteremia could not be observed and a combination of host factors and fine-tuned bacterial gene expression patterns likely determines disease outcome.

Task 3.3 Functional validation of implicated traits.

Bacterial NSTI isolates (S. pyogenes, S. dysgalactiae, S. aureus) were tested for their pathogenicity in appropriate human cell lines. S. pyogenes invasion into endothelial cells revealed M-type associated differences in invasion potential of S. pyogenes NSTI isolates. Interestingly, the predominant M1 serotype isolates displayed the lowest invasion potential indicating epithelial cell based persistence and spreading to be of the minor importance for pathogenicity of NSTI isolates. Partner 1 reported the importance of neutrophils in disease pathology (Snäll et al Sci Rep 2016; Uhlmann et al J Infect Dis 2016). Analyses showed that S. pyogenes strains were particularly potent triggers of neutrophil activation and degranulation, in particular by SpeB-negative strains. Further analysis, identified phosphoglycerate kinase as a stimulatory factor. This finding is of interest in light of reports of hypervirulent SpeB-negative S. pyogenes variants present during invasive infections, also found in NSTI patient biopsies (WP5, partner 1).

The analysis of S. aureus toxin-mediated tissue pathology revealed that PVL and α-toxin both contribute to tissue damage in cell specific manner (Partner 1 and 10). Of importance, toxin-mediated epithelium destruction could be inhibited by IVIG containing antibodies against α-toxin and PVL.

In order to decipher the respective role of S. pyogenes and S. aureus in the pathogenesis of necrotizing fasciitis and myositis, the virulence of the two species was compared in human keratinocytes and myoblasts (partner 10). S. aureus isolates from NSTI exhibited strong adhesion and internalization rates into human keratinocytes and specifically into myoblasts. S. aureus from NSTIs also exhibited extraordinary cytotoxicity toward myoblasts which correlated with high levels of psmα and RNAIII transcripts. These findings suggest the unique property to invade and kill myoblasts to contribute to S. aureus NSTI severity.

Task 3.4 Functional profiling and identification of host traits/pathways contributing to tissue pathology and/or systemic toxicity.

We established a serology screening approach to identify risk factors for the development of a severe NSTI. We purified all 11 known streptococcal superantigens and investigated the role of toxin-specific antibodies in NSTIs caused by S. pyogenes and the potential beneficial effect of IVIG treatment. We identified a state of serologic susceptibility in NSTI patients during the earliest stages of the infection. Thus, all studied NSTI patients exhibited a deficiency in specific antibodies directed against the causative S. pyogenes strains and the majority of their exotoxins during the initial stage of the infection. We also showed that the clinical application of IVIG during the course of infection compensates the observed antibody deficiency, but is unable to
halt the disease progression, once tissue necrosis has developed. These observations emphasize the requirement of pre-
existing pathogen-specific antibodies to prevent the irreversible progression of tissue infections into severely spreading NSTI
and urge further investigations on the beneficial effect of IVIG-based early phase intervention strategies to prevent the severe
effects of this devastating bacterial infection.

We established a protocol for the purification of RNA, DNA and proteins from infected tissue samples, which was used to
generate RNAseq data of 117 tissue samples from 91 patients (Partner 8). For 47 of these RNAseq datasets that contained a
high amount of reads originating from both, the host as well as the infecting pathogen(s) we performed the quality filtering
and mapping against the human genome and against a set of bacterial genomes guided by 16S rDNA based amplicon
sequencing. All analyzed tissues were characterized by an acute inflammatory signature, however, our analysis showed
significantly different gene expression signatures in monomicrobial Streptococcus spp. versus polymicrobial NSTI. The core
inflammatory signatures comprised genes encoding important inflammatory mediators and key inflammatory mediators were
also detectable in plasma. Detailed analysis of the genes differentially expressed between streptococcal and polymicrobial
NSTIs showed a strong interferon-mediated immune response in patients infected by Streptococcus spp. Respective proteins
could also be identified in plasma of these patients. Polymicrobial NSTIs in contrast were characterized by a significantly
higher expression of host genes encoding extracellular matrix components. Thus, distinct transcriptional signatures within the
infected tissue distinguish streptococcal from polymicrobial NSTIs.

Analysis of transcriptomes also indicates human pathobionts and pathogenic streptococci to utilize different strategies for
nutrient acquisition during infection. Whereas streptococci use free carbohydrates, polymicrobial communities exploit host-
derived proteins, peptides and amino acids. Besides different using nutritional strategies, differences were also observed in
the virulence gene profiles. The transcriptome of Streptococcus spp. revealed genes encoding virulence factors as highly
expressed during NSTI and clearly adhesion/invasion, immune modulation, proteolysis and toxin producing activities
contribute to the pathogenicity of Streptococcus spp. NSTIs. In accordance with the diversity of the polymicrobial community
a high diversity of virulence-associated domains was expressed in polymicrobial NSTIs. However, the virulence associated
domains expressed were restricted to those mediating cellular adhesion and extracellular proteolytic activity. Moreover
detailed analysis of the transcriptional profiles of distinct genera in polymicrobial NSTIs showed that they contributed to
different extent and with different functionalities to the overall community pathogenicity. Thus, while pathogenic streptococci
express a wide range of virulence factors that mediate the different steps of infection comprising colonization and evasion of
the host immune response, the pathogenicity of pathobionts is dependent on the complementary activities of multiple
bacterial genera, which enhances the virulence of the bacterial community. These differences result in distinct patterns of
molecular host pathophysiology. Consequently, a rapid and accurate microbial diagnostic is necessary to optimize clinical
treatment and to tailor intervention strategies towards the specific, etiology-dependent molecular pathophysiology.

4.1.3.4 WP4 Systems modelling and integration

The objectives of this WP were to:
® identify and unravel -both in the host and the pathogen- key molecular networks underlying NSTI;
®to develop a modular, physiopathological model framework capturing the main sets of host-pathogen interactions in the
onset of disease; and, on the basis thereof,
   – to identify biomarker sets of multiple nature for early diagnosis and novel therapeutic targets for intervention.

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The results achieved in this WP within INFECT are described below and relates to the WP specific Tasks:
Task 4.1 Data standardization and data management.
To handle the heterogeneity of the omics data generated through the infect project a Semantic Annotation Platform with Provenance, call SAPP, was developed. The goal of this platform is to handle various kinds of omics data and to integrate them to into a single multi-omics resource. The basis is built upon a Genome Ontology (GBOL) allowing to persistently store high-quality genomics data while making the entire resource FAIR. Through an integrated SPARQL endpoint we were to make the resource human as well as machine interoperable. Through this query language, all data becomes accessible through either an open or a private channel making it more efficient to find and reuse specific data for different research topics. Within INFECT, it enabled us to analyse the data obtained throughout the project and perform in-depth analysis on the host and the microbial environment in an efficient manner while preserving all information. This allowed us to further investigate host-pathogen interaction using different applications.

Task 4.2 Defining a blueprint of the host-pathogen interaction networks in NSTI.
The dual RNASeq from 106 patient biopsies (a concept and strategy proposed by partner 9 and implemented by Partner 8 in WP3) was mapped against both human and bacterial genomes to obtain gene expression of both the host and resident pathogens. The tool Kallisto was used to map the sequences against the human genome (GRCh38 release 91). The same sequences were mapped against several bacterial genomes using the published pipeline HUMAnN2. We found the most highly expressed genes in the patient biopsies to be involved in metabolic processes and in immune processes. Concerning pathogens we found no major genera of bacteria that are shared by all the biopsy samples. The 10 most abundant genera are Streptococcus, Escherichia, Parvimonas, Peptostreptococcus, Porphyromonas, Propionibacterium, Solobacterium, Bacillus, Prevotella and Filifactor.

The gene expression of the host and that of the pathogens were integrated using multivariate approaches, providing information about the host response to certain bacterial groups or functions. All the 106 samples were used for this integration to build an interaction network, which is highly clustered into sub-networks, with several bacterial genes correlated with one human gene. Analysis of the bacterial genes show that 63% of the bacterial genes in the network are from the species Streptococcus pyogenes which is a known pathogen implicated in NSTI. For instance, there are 5 human genes (NAMPTP1, SRGN, IL18R1, ZPR1, ZNF302) that correlate with this species. The S.pyogenes genes in the three clusters correspond to different functions such as biosynthetic processes which could be important for biofilm formation. The process of forming a biofilm is being investigated by partners 1, 9 and 14 for being an important defence mechanism of the invading pathogens. The human gene correlated with this process is PEAK1, which is known to modulate cell adhesion and is responsive to bacterial load. Several manuscripts exploring host-pathogen interactions are being prepared at the time of reporting.

Data from those 106 biopsies for which gene expression was made available were further analysed to look for any differences in the host response to the NSTI in respect to the presence of comorbidities, in particular type 1 and 2 diabetes. Based on various factors (such as ages, sex, surgery) 17 NSTI patients who do not have diabetes were matched for comparison. We did not find significantly differentially expressed genes but on a gene co-expression network level, we found striking differences between the two groups. Co-expression networks were built from gene expression profile of diabetic and non-diabetic NSTI patients and network modules were then analysed for functional information. We found several modules having similar functional properties even if the genes involved were not the same, indicating dysregulation of the molecular machinery. A module of interest was one pertaining to the GO term ‘Striated Muscle Contraction’ which is of interest in the context of diabetes because it is known that muscle functions and physiology are altered in diabetic patients.

Task 4.3 Dynamic models of progression of infection and immune defense.
Partners 9 and 14 have deployed a genome-scale metabolic model for the mammalian host as well as the modeling of specific regulatory aspects of the host. A predictive cell-specific model has been realized that can be used to generate valuable predictions. Suitable approaches to handle missing data and data pre-processing were implemented in collaboration with WP2 to ensure the best quality of the final dataset.

Generic metabolic models were also built for the following species based on their genes sequence: Human generic model, Mouse generic model, S. pyogenes generic model together with generic compartment based community models for Human (bacteria community model) and Mouse (bacteria community model).
Gene expression data from biopsies from NSTI patients were used to build NSTI vs. normal specific metabolic models for all the generic models we generated before for a total of more 100s of metabolic model which were compared between the generic and the specific metabolic models. Using the flux variability analysis algorithm, we listed the differences in the fluxes of the different species combinations in each of the samples and compared them with the generic metabolic models possible fluxes; results are being interpreted.

To simulate the biofilm formation of S. pyogenes we built tools to simulate and graphically show the biofilm formation in a 3D cellular automata model (Partner 14). To perform the simulations we used our developed media prediction tools (KOMODO and the growth media prediction tools) we used these media to give predictions based on the generated model and perform manual curation of our S. pyogenes metabolic model. The analyses were supported by multivariate statistics (Partner 9).

Statistical algorithms have been tailored for identification of patient-bacteria/treatment associations in clinical record data. Two statistical analysis plans have been published to test hypotheses generated in WP2, one on overall study of the cohort with a particular interest on death outcome and subset of patients with diabetes. The different statistical models used were survival analysis, logistic regression and linear model.

Task 4.4 Building a modular, pathophysiological modelling framework of the host-pathogen signatures.

A death prediction model developed jointly by Partners 11 and 14 was developed using selected “early” clinical variables, like baseline, demographic and early measurements. From those, a set of best predictor variables were selected using Random Forests. For the actual prediction different machine learning tools were employed: Random Forest as well as Support Vector Machine. The prediction model was included as a feature for a prototype mobile app, which allows users/clinicians to input the values for a small set of predictive early parameters in order to receive a probability of patient death/amputation after a certain time period post diagnosis, e.g. after 30 days. In another approach, the set of clinical variables was combined with gene expression data from the RNA-Seq experiments, though with less success than using only clinical parameters.

During the 5 years of the project, Partners 9, 11 and 14 have developed multiple bioinformatic tools to handle, visualise and investigate bacterial data and information. In addition to the above-mentioned applications SAPP, GBOL, SynDI, tools developed were:

NInA (Network Integration and Analysis) - a tool for synchronous visualization of multiple biological networks and pathways in NSTI-specific context to facilitate understanding the molecular complexity of the disease. MTA (Metabolism Transformation Algorithm) – an algorithm that builds tissue/condition specific models based on Gene Expression. Gene expressions were the data collected in the INFECT project in cases of NSTI; EDGE – a research to predict the functional implication of over expression of metabolic genes, we planned to use this research in predicting possible situations within an NSTI situation. KOMODO (Culturing the unculturables) - an algorithm that assists in finding growth media for unculturable and difficult to grow bacteria. Predicting growth media for bacteria – using metabolic modeling finding minimal media that can enable growth of bacteria since we expected that some of the bacteria we would encounter will be hard to grow in-vitro and we thought of using this algorithm to assist with that together with the next research listed. Gene promiscuity prediction – Gene promiscuity is one of the methods of bacteria to gain antibiotic resistance. Knowing which gene’s promiscuity can enable such resistance is important when suggesting antibiotic treatment.

Furthermore, Partner 9 has developed and refined a number of computational and statistical methods and models to be deployed in the analysis of the multidimensional data generated in WP3 and analyzed in WP4. All these are listed in Table 4. Altogether, the various models and analyses developed in WP4 have enabled to build a modular framework that was (and will be, beyond the duration of the project) pivotal to ascertain the host-pathogen interactions as measured in the experimental and clinical WPs and to pin-point and uncover underlying mechanisms in the clinical context.

4.1.3.5 WP5 Host-pathogen interactions at the tissue site of infection (NSTI tissue biopsies)

The objectives of this WP were to validate results obtained in WP1-4, and to identify mechanistic action of novel therapeutic strategies. This was done by partner 1 through analyses of host-microbe interaction at the local site of infection, i.e. in patient tissue biopsies, in particular to:

- test model-driven hypothesis and data on host and pathogen traits from WP1 and WP3-4 by detection of implicated factors in patient tissue biopsies
determine mechanistic action of the therapeutic strategies HBO and IVIG by analyses of patient biopsies pre- and post-therapy elucidate whether there are different host-pathogen interactions in NSTIs caused by varying pathogens.

The results achieved in this WP are described below in relation to the WP specific Tasks. The results are based on analyses of patient tissue biopsies collected from enrolled NSTI patients in WP2. As WP2 identified the main causative agents of NSTI to be S. pyogenes, but also group G Streptococcus (SDSE) were a common cause, as well as a few S. aureus cases. Therefore, the WP5 analyses have focused on immune-stainings and analyses of the biopsies from patients infected with S. pyogenes, group G streptococcus (SDSE), and S. aureus; in total WP5 has analysed >180 biopsies.

Task 5.1 Assessment of predefined bacterial and host traits hypothesised to be involved in NSTIs.

A panel of markers for bacterial load and host inflammatory responses was selected based on previous publications (Thulin et al PLoS Med 2006; Sundén-Cullberg et al Crit Care Med 2005) in which these factors had been implicated as markers of severity of infection. The immunostaining data were analysed in relation to microbiologic aetiology, disease progression and outcome as well as in relation to treatment protocol (further elaborated on in task 5.4).

The results showed that there are variations in different biopsies but there were no significant differences in tissue severity (here defined by HMGB1 levels) or inflammation (here defined by IL8 levels) between the different pathogens. The data illustrates how varying the host response is in individual NSTI even when categorized dependent on microbial aetiology; supporting the concept of patient stratification based on host immune status. The results showed a trend to lower inflammation, yet equal tissue severity, in S. aureus infected tissue was noted. This supported our hypothesis that the pathophysiology is microbe-dependent, and was further explored in WP3.

A key study of WP5 here was that of HMGB1 as a potential novel marker for NST1 severity. Partner 1 demonstrated through an analyses of NSTI biopsies and compared these to the uncomplicated tissue infection erysipelas. The data showed that HMGB1 significantly correlate with degree of inflammation and severity of infection. Taken together, the findings provide the first in vivo evidence that HMGB1 is abundant at the local site of bacterial soft tissue infections and its levels correlated to severity of infections; hence, indicating its potential as a biomarker for tissue (Johansson et al Front Cell Infect Microbiol. 2014).

Task 5.2 Identification of novel bacterial and host protein determinants in tissue identified in the integrated model.

Key findings of this task include:
- Contribution to the study headed by partner 15 (WP1) where systems analyses revealed L1β as a key determinant of NSTI severity in mice. Partner 1 used gene expression analyses of infected patient tissue as well as protein analyses of plasma samples and thereby provide data supporting patient data that strengthened the clinical relevance of the murine data. (Chella Krishnan et al. PLoS Pathogens 2016).
- Analyses of clinical data implied a potential biofilm problem (WP2). Through analyses of patient biopsies (WP5), partner 1 could provide evidence of S. pyogenes biofilm formation in NSTIs. This emphasized the urgent need for biofilm to be considered as a potential complicating microbiological feature of GAS NSTI and, consequently, reconsideration of antibiotic treatment protocols. (Siemens et al. J Clin Invest INSIGHT. 2016). This is one finding of INFECT that resulted in changed clinical practice!
- Comparison of RNA-seq data of biopsies from S. pyogenes NSTI patients and skin from healthy donors identified a large number of significantly differentially expressed genes (WP3,WP4, partners 1, 8, 9, 11). Reactome pathway analysis on this gene set identified a set of over-represented immune system. Notably, the same host signatures were significantly overrepresented in infected vs uninfected skin tissue model (WP6, partner 1), as well as in the infected murine model (WP1, partner 15).

The signatures were further verified through a multiparameter imaging workflow established by partner 1 in WP5 to enable phenotyping of immune cells at the tissue site. Staining of patient biopsies and infected skin tissue model (WP6) provided
mechanistic insight to the implicated signaling pathways (Snäll et al, manuscript in final preparation).

- Neutrophil degranulation was implicated in the RNAseq analyses (WP3, WP4), which was exciting in light of the studies in WP5 (partner 1) demonstrating neutrophils as key players in NSTI and in S. pyogenes NSTI in particular (Snäll et al Sci Rep 2016; Uhlmann et al J Innate Immunity 2016), including also identification of a novel streptococcal factor PGK with potent neutrophil stimulatory capacity (also relating to task 5.3) (Uhlmann et al J Infect Dis 2016).

Task 5.3 Tissue expression of bacterial virulence factors (both protein and gene expression).

- Genes encoding for selected bacterial factors, i.e. the cysteine protease SpeB, streptolysin O (slo) and S (sagA), streptokinase (ska), and the M-protein (emm), as well as key regulatory systems (RofA, Mga, Ihk/Irr) were assessed by qRT-PCR analyses of tissue biopsies of NSTI patients, many of which were upregulated in the tissue setting (WP5, partner 1). (Siemens et al, J Clin Invest INSIGHT, 2016; Siemens et al, Sci Rep 2015). The results were of importance for the understanding of which factors/regulatory systems promotes biofilm formation and or cytotoxicity resulting in tissue damage. Siemens et al Sci Rep 2016, reports that the cytotoxin SLO is a key determinant of tissue damage associated with SDSE NSTI (WP3, WP5 and WP6).

- The role of SpeB in infections has long been debated. This is due to the fact that this important protease degrades several important host proteins but also endogenous virulence factors. Here we studied this through culture of tissue biopsies directly on casein plates, which readily identifies colonies expressing SpeB or not. The data showed that all biopsies contained both SpeB+ and SpeB- negative variants identifying a phenotypic variation in the same infectious site (Siemens et al J Clin Invest INSIGHT 2016). This is of interest in light of partner 1’s studies on neutrophil activation and the identification of a novel neutrophil activator PGK, which proved to be susceptible to SpeB degradation (WP3).

Task 5.4 Documentation of therapeutic efficacy.

For this task analyses of snap-frozen tissue biopsies collected pre- and post-IVIG and/or HBO treatment were done to evaluate whether there are variations in host and bacterial factors that might be contributed by the treatment. As there are many potential confounders (among others the fact that the tissue is from necrotic sites involving different tissue types, different surgical sites and collection on different days post enrollment) in this analyses, the results can only provide an indication of mechanistic action. A large percentage (77%) of the stained S. pyogenes infected NSTI patients had received IVIG as well as HBO (74%). Treatment was started during day 0 or 1. HMGB1, IL8 and bacterial load are shown in patients categorized to treatment with IVIG/HBO (100% received IVIG, n=3, 10% did not receive HBO) versus no IVIG (2 also received HBO, 7 did not). The data show that there was no difference in HMGB-1, IL8 or bacterial load over the treatment days. Samples from the same patient were also analysed in a matched analyses, but also here we did not see any differences over time. This should not be interpreted as the treatments having no effect but likely that the tissue biopsy collection was too diverse to allow for this type analyses, as the tissue collection protocol (and ethical permit) only allowed for collection of tissue that was necrotic and needed to be surgically removed. Hence, a tissue area that had improved and had no necrosis could not be collected.

4.1.3.6 WP6 Artificial tissue modeling

The objectives of this WP (Partner 1) were to:

- Elucidate the role of the well-defined bacterial toxins, e.g. Superantigens, cytotoxins, streptolysins, PVL, α-toxin etc., in the pathogenesis of NSTIs.
- Identify and confirm tissue-specific bacterial disease traits.
- Confirm and validate patient and murine data regarding pathogenic mechanisms and factors contributing to severity of NSTIs.
- Test novel therapies (HBO/IVIG) in order to obtain data on dosage and mechanistic actions.
- Identify novel molecular targets and test their therapeutic potential.

To be able to address the WP6’s objectives we initiated the work by establishing an organotypic tissue model resembling human skin. This important milestone was achieved at an early stage of the project (D6.1 MS24). The model proved useful for modelling of infection with streptococcal and staphylococcal strains collected in INFECT. 1 x 106 colony forming units of bacteria was found to be optimal for infections up to 48 hours, leading to moderate-severe tissue damage. At different time
points, 8, 24 and 48 hours post infection; tissue culture supernatants, as well as non-infected or infected tissue models were harvested and subsequently processed for histology and immunofluorescence analyses, protein detection, RNA sequencing and bacterial growth.

A summary of the key findings of WP6 (Partner 1) is described in relation to the WP specific tasks:

**Task 6.1 Identifying toxins involved in NSTIs**

Using Streptococcus dysgalactiae subsp equisimilis (GGS) isolates to infect the skin tissue model, revealed that three invasive NSTI strains as well as one non-invasive strain derived from an uncomplicated wound infection, were all able to colonize and replicate in the model tissue. While the three invasive strains caused severe tissue damage characterized by substantial epithelial disruption and detachment, which significantly increased over time, the non-invasive strain induced mild to moderate epithelial disruption. Furthermore, higher SLO activity was identified in invasive, as compared to non-invasive strains, whereas the reverse was true for SLS activity. A positive correlation between SLO activity and keratinocyte cytotoxicity was found. Thus, pointing towards important differences in virulence between GGS isolates potentially dictating disease outcome (Partner 1, 6) (Siemens et al, Scientific Reports, 2015).

Differences in cytotoxicity was also observed between Staphylococcal isolates, as evident by the relatively sever tissue damage caused by S. aureus strains harboring tyrosine in position 223 of AgrC. Thus, a naturally occurring single amino acid substitution (tyrosin to cysteine) at position 223 of AgrC determines starkly different S. aureus virulence phenotypes, e.g. cytotoxic or colonizing, as evident in both in vitro (partner 1, 10) and in vivo (partner 1, 15) skin infections (Mairpady-Shambat et al, 2016, Scientific Reports).

**Task 6.2 Dosage and mechanistic action of therapeutic strategies**

In WP6, the two main therapeutic strategies to be tested were hyper baric oxygen (HBO) and human intravenous immunoglobulin (IVIG) treatments. The skin tissue model tolerated the HBO procedure, and this work will continue post INFECT to evaluate the effect of HBO, by the analyses of in vivo (patients) findings in combination with in vitro experiments. To test the effects of IVIG on bacterial exotoxins, we first preformed infections of a well-established 3D human lung tissue system (previously developed by partner 1) with S. aureus isolates in the absence and presence of IVIG. This showed that IVIG potently neutralize S. aureus exotoxins and reduce the tissue damaging properties of S. aureus (Partner 1, 10) (Mairpady-Shambat et al, 2016, Disease Models and Mechanisms). This experimental set was transferred to the skin model, and we investigated the efficacy of IVIG to reduce the cytotoxic activity of GAS and GGS-derived exotoxins. This revealed that IVIG partly reduced, in a concentration dependent manner, the cytolytic effects of GAS-derived exotoxins, but had little effect on GGS-derived exotoxins. Taken together, this indicates that the cytotoxicity mediated by GAS can be targeted by IVIG. However, this needs to be investigated further, and the clinical data analysed in this respect (ongoing studies).

**Task 6.3 Tissue modeling and systems biology**

The first version of tissue model contained human skin keratinocytes and fibroblasts, and was therefore further developed to include also human monocyte-derived macrophages, which are of critical importance in the pathogenesis in bacterial infections. These models were infected with different INFECT GAS isolates, and subsequently processed for RNA sequencing to generate transcriptomic data on human skin infected for different length of time (Partner 1, 8, 9). Transcriptional profiling of S. pyogenes (GAS)-infected vs non-infected skin tissue models revealed alterations in inflammatory signatures, similar to that identified patient tissue biopsies (WP5) (Manuscript in preparation). This unique set-up will be used to gain further knowledge of changes in gene-, protein- and metabolite- expressions during Streptococcus and Staphylococal infections.

**Task 6.4 Host determinants of inflammation.**

Using the skin tissue model Partner 1 investigated the inflammatory course during S. pyogenes infection. Since the transcriptional analysis and the subsequent bioinformatic analysis suggested that macrophages are involved in and/or alternatively affected by the course of infection, partner 1 (WP5 and WP6) established the ability to study macrophage's
phenotype / functionality with multiparameter imaging at the tissue site. Confocal microscopy analyses of infected 3D skin tissue models revealed a shift in inflammatory cellular status.

The usages of skin models to recapitulate NSTI infections also showed that IL1 beta (mRNA and protein) was increased in infected skin models compared to uninfected skin models. This finding verified the gene expression analysis of S. pyogenes-infected mouse tissue performed by partner 15 and that identified IL-1 beta as a key regulator likely to contribute to NSTIs (WP1, PLoS Pathogens, 2016). IL-1 beta was also confirmed to be upregulated in skin tissue and plasma of NSTI patients (WP2 and WP5, partner 1, 2). Together this has led to the identification of the IL-1 beta network as a key network involved in modulating the differential susceptibility to NSTIs (Chella Krishnan, et al., PlosPathogens, 2016).

The skin tissue model was also instrumental in dissecting the properties of S. pyogens to form biofilm in skin (WP2, WP5, WP6, partner 1, 2, 6). The Nra gene regulator was found to be one key component (Siemens et al., J Clin Invest INSIGHT, 2016) in biofilm formation. Biofilm was verified in patient tissue biopsies and associated with massive bacterial load, and a pronounced inflammatory response, as well as clinical signs of more severe tissue involvement. This novel finding of GAS biofilm formation in NSTIs emphasize the urgent need for biofilm to be considered as a potential complicating microbiological feature of NSTI and consequently has led to change in clinical practice.

Further exploring determinants of infection, Partner 15, found evidence that adipocytes, important for metabolism, are infected with S. pyogenes in the experimental model of NSTI. In addition, metabolomics analysis of INFECT plasma samples (WP3, partner 1, 9) indicated alter metabolism during infection. Therefore, partners 1 and 15 initiated a collaboration on exploring the role of adipocytes in the inflammatory response in NSTI. As a result, a skin tissue model was developed which supports introduction of adipocytes. Utilizing these 3D skin tissue models, will enable partner 1 and 15, to address the role of adipocytes in the pathogenesis of NSTI. Together this demonstrates how amendable this model system is and can be modified based on different needs by the users.

Main achievements/impact of WP6:
- Novel tool for biomedical research
- Recapitulating infection for the generation of gene, protein and metabolite expression
- Enable testing of therapeutic strategies in a human setting
- Customization – different cell types can be introduced
- Verification of in vivo findings
- S. pyogenes biofilm – awareness, bacterial persistence, treatment, antibiotic stewardship
- IL-1 beta as a key network in NSTI – novel treatment target

4.1.3.7 WP7 Translation of results into prototype for novel diagnostics
Translation of obtained results (clinically relevant pathogens and pathogenic disease traits) into a clinical compliant diagnostics approach by applying compact sequencing (pathogen detection – DNA) and compact profiling (disease traits – antigens) – both multiplex diagnostic technologies. The tool will be designed to support therapeutical decision as for example antibiotics treatment, decision as for example antibiotic treatment, decision for surgery or closing wounds.

The results achieved in this WP are described below and relates to the WP specific Tasks:

Task 7.1 Test specifications.
All major requirements from clinicians have been collected and summarized in short (preliminary) product specifications which served as guidelines for test development, discussion with interested clinicians or the interested public.
Partner 12 (Anagnostics Bioanalysis GmbH), as the WP leader at that time (later replaced by Cube partner 16), intensively communicated with all clinical partners (2,3,4,5,6) to outline general requirements for diagnostics in case of (suspected) NSTI. All hospitals (except partner 5) were personally visited partner 12, the situation on-site evaluated and the project partners
interviewed. These personal interviews delivered a clearer picture on what procedures and efforts are acceptable in clinical practice and what questions should be answered. Taking into account technical feasibility and experiences of partner 12, initial specifications were outlined (partly as assumptions).

Even if the list of pathogens, the list of necessary antibiotic resistance markers and inflammation markers was to be expected to be extended or altered during the course of the project, the cornerstones of diagnostics tests have been set out in an specification document.

Task 7.2 Implementation of test prototypes.

After specifying the diagnostic tests to be implemented (see above), an iteration of design, implementation and testing (preliminary verification) was executed. Along with the development of the tests themselves, the underlying assay technologies have been (further) developed as well.

The work itself fell into bioinformatics (design of microarray probes and PCR-primers), material testing (above all various antibody – antigen combinations, antibody – secondary antibody combinations), method development (protocol development), (further) development of hard- and software (Cube developed a new device between 2016 and 2017), development of production technologies (coupling of molecules on hybcell surface, printing of microarray onto hybcell surface, drying of reagent mix into cartridge, etc.). So, the work was mainly work related to laboratory experiments.

Along with the development (as a regulatory requirement) a proper documentation of the development and its results – including risk management - had to be done.

To be prepared for verification and later clinical validation, 0-series of the (test)kits had to be produced (figure 7.1). Example of testkits developed and produced ready for verification (Pathogens xA); appendix). First, the DNA-based tests for pathogen ID and the biomarker test (in an preliminary version) have been developed.

Later, after Cube partner 16 took over, new versions of the tests had been developed (broader panel of pathogen ID test (hybcell Pathogens DNA xB); resp. patientmonitoring test with a slightly changed set of markers, with a reduced number of pipetting steps (only pipetting sample into cartridge) calibrated for whole blood to enable the application of the technology on the intensive care ward itself (figure 7.2 Usage of the biomarker test: just pipetting of 100µL whole blood into the hybcell cartridge.)

After the above mentioned iterations, the test panels offered as either hybcell Pathogens DNA xB or hybcell Patientmonitoring Blood xA are summarized in Figure 7.3 (Tested pathogens (bacteria, fungi) and resistance genes of hybcell Pathogens DNA xB ; appendix) and 7.4 (Tested biomarkers (inflammation, organs, coagulation) of hybcell Patientmonitoring Blood xA; appendix).

Task 7.3 Verification of test prototypes.

Within this task the basic analytical features and the requirements of the specification document(s) of these tests have been tested and verified by Cube (partner 16). The result of this verification was summarized in (verification) reports. Based on these verifications, the tests were improved if necessary and finally released for further clinical validation.

Task 7.4 Clinical validation of tests.

155 plasma samples collected by partner 2 (RH) have were tested with the hybcell Patientmonitoring Blood xA and comparative tests by partner 2 (RH). For the parameters CRP, Cystatin C, Myoglobin, NGAL and PCT, already established tests have been used for comparison. For these biomarkers, the correlation was acceptable to good (Figure 7.5; appendix). For the other biomarkers ELISA products were purchased and established by the RH laboratory. No satisfactory correlation could be established.

Further 147 plasm samples samples from the INFECT biobank (from partner 5 (SU) and partner 6 (UiB) have been tested. The collective was basis for clinical examination: Prognosis of acute kidney injury (AKI):
All biomarkers (and combinations) have been examined with help of a ROC (Receiver Operating Characteristic) curve, how they would prognose acute kidney injury (AKI).

Myoglobin showed the highest AUC for a single marker, Myoglobin and Cystatin C (as combination slightly improve the AUC (from 0.85 to 0.86).

Prognosis of mortality:
The SOFA score shows the highest AUC, followed by the markers myoglobin (cardiac / muscle destruction marker). A combination of markers does not improve the AUC.

From sample to result, cost and ease of use:
The test has a turnaround time of approximately 13 minutes (for all markers), and the cost is € 33 (=9 markers). The usage on the intensive care unit seems feasible.

Task 7.5 Compilation of clinical results.
A public report has been compiled from the clinical results obtained mainly in task 7.4.

4.1.3.8 WP8 Dissemination and exploitation
The ultimate objectives of WP8 was to ensure an efficient dissemination and exploitation of knowledge generated within the INFECT project, the specific aims of WP8:
- prepare and update information for the external open access website
- disseminate clinical guidelines
- disseminate scientific advances to researchers and SMEs
- prepare information material for patients, medical staff and society in large
- provide training for medical staff

The ultimate objectives of work package 8 (“Dissemination and Exploitation”) was to ensure that the knowledge produced within INFECT was efficiently disseminated (all partners). This has been achieved by using a variety of dissemination activities as detailed in tables A1 and A2. A few key achievements of this WP are described below in relation to the WP specific Tasks:

Task 8.1 and T8.2 Dissemination of information of the INFECT consortium and project, and Dissemination of knowledge generated in INFECT
Within the first weeks of the project, and well ahead of schedule, an open access external web site was created and opened for the public. This website has been regularly updated.
■ At the same time an informative leaflet to be used e.g. at meetings with health care workers, scientists, decision makers, patients and relatives, was produced.
■ All partners have throughout the project period presented their scientific advances through presentations at scientific meetings, patient organization meetings, lectures, scientific publications accepted by well-renowned peer reviewed international journals. Also the INFECT project as such has been presented at several national and international conferences.
■ Establishing guidelines (D8.6). It was only into the study period it was realized that medical guidelines based on the novel scientific knowledge produced by INFECT must be created by an independent third parties, in order to be acknowledged, supported and advocated by professional communities such as medical societies. The consortium has established contact with clinical association (e.g. SSAI) with the intention of establishing (national) guidelines.
■ The achievement of the entire INFECT project will be summarized in a book invited by Springer Nature publishing group (edited by partner 1, and co-edited by partner 6; all partners contributing).
■ A YouTube video describing the achievements of INFECT is being produced and will target the society at large, estimated release autumn 2018. Partner 6 is together with DigiUiB, the media department at UiB responsible for this work.

Task 8.3 Training of medical staff
■ This will be offered as a 1-day workshop on NSTI held in association with the Scandinavian Society of Anaesthesiology and Intensive Care Medicine (SSAI) in conjunction with the SSAI 2019 Congress to be held in Copenhagen August 2019. The venue for the one day course will be in the National Hospital, Copenhagen, Denmark, and it will be held one day prior to the conference, at August 27th 2019. Announcement of the conference is in development and the SSAI will ensure the timely
The YouTube video described above will promote general awareness of the importance of NSTI, which although targeting the society at large will be useful also for medical staff.

4.1.3.9 WP9 Project management during the period
In summary, the project management activities aimed to achieve/provide:
- an efficient management and administration of the project in line with legal and EC regulations.
- a functional communication between the participants in the project.
- a robust management component to efficiently communicate with the EC.
- support to the project partners regarding administration and reporting.
- identification and handling of problems at early stages.
- tools to monitor and disseminate project progress and results
- productive discussions with advisors and within the Project Steering Committee.

The INFECT consortium is composed of a team of multidisciplinary researchers, clinicians, SMEs and a patient organization, each with a unique expertise, technical platform and/or model systems that together have the means to successfully conduct the research proposed. There are in total 14 partners from 11 different countries that have worked together to achieve the goals set forth in the 8 distinct, highly interrelated, scientific WPs.

A critical component of INFECT has been to fully exploit the multidisciplinary expertise and resources provided by the different partners. Data-sharing has been critical but equally important has been the sharing and exploitation of partners’ respective expertise that is needed for optimal data utilization. WP9 has focused on achieving this through a variety of actions (outlined below), as in our view, this is an absolute requirement for a systems medicine approach to be successful, and therefore, this has been highly prioritized in INFECT. The leadership and coordination of INFECT has been based on the principle to always ensure that the actions taken are aligned with the overall aim of the INFECT project, namely to generate advancement that will benefit NSTI patients.

Task 9.1 Management of INFECT and Task 9.2. Coordination of INFECT activities
Key activities have included:
Consortium meetings held annually as well as a final meeting. These meetings have included: scientific reports by all partners, presentations of data, discussion of problems and actions needed, as well as ongoing work related to the WPs. Also partner 1 has used to opportunity to inform about administrative tasks, such as reports, deadlines, etc.

The INFECT advisors (Prof Reuss and Dr. Morgan) have been invited and attended all these meetings. This has been most valuable as they have provided important feedback to the consortium and also to the commission as they performed the mid-term review and always have provided short summaries from the annual meetings. See end of WP9 section for their comments from the final INFECT meeting held in Bergen, June 2018.

Project steering committee meetings has been held annually in conjunction with the annual meetings to discuss INFECT progress and critical issue.

Providing support in planning and guiding the project. This has been done through continuous contact with partners through email correspondence, Skype contacts, and meetings both initiated by the coordinator and by the WP-leaders. Part of these activities aimed to address challenges within the project as soon as they raised.

Administration and distribution of the EC funds to partners. No major deviations and actions according to the grant agreement and associated amendments.

Follow-up on work progress in respective WP. This has been done through meetings, mail and phone communications, and review of deliverable reports submitted for respective WP.

An important management tool has been the periodical (every 6 month) DIP (data integration panel; DIP) report. The purpose of the DIP has been to oversee and ensure the effective creation, management and sharing of samples and data within the consortium, as this would enables fully exploit the potential of INFECT. At regular occasions (meetings, TCs, Skype, email,
phone), we discussed which samples and data to focus our analysis and modelling efforts on, to ensure that we work towards the objectives of INFECT, as well how to improve generation and sharing of data. The DIP reports have reviewed the status of patient enrolment and sample shipment, as well as data generation, analyses, processing and sharing. Overall, the aim of the DIP has been to oversee the current status of progress within INFECT and to ensure that INFECT samples and data became accessible to all partners as needed and agreed.

Compliance with reporting duties to the commission. Annual and financial reports have been reviewed and submitted on time.

Additional meetings between/within WPs has occurred continuously. In addition to the meetings listed above, many interactions took place through regular email/skype and TCs. Coordinating and ensuring administrative information dissemination to partner members by email, website and to the EC project officer. This has been done throughout without any major issues arising.

Ensure full integration and coordination of the research teams. As needed, necessary contacts have been taken between involved partners. Communication has been through regular Skype, mail or telephone to deal with smaller urgent issues. This has during this period been sufficient to ensure efficient progress and problem solving.

Task 9.3 Dissemination of INFECT.

Due to the central issue of dissemination in INFECT, a special WP (WP8) has been responsible for dissemination and exploitation of the results gained in INFECT (See WP8 report). Dissemination activities have been several and of excellent quality. To give a few examples:

1. The INFECT consortium has received a lot of attention in the scientific community, to name a few highlights:
   a. The 20th International Lancefield meeting on Streptococcal infections, September 2018: Partners 1, 6 and 8 participated with INFECT presentations and all received oral presentation, including the coordinator Norrby-Teglund who was invited as plenary speaker. In the concluding remark, the organizer highlighted the INFECT project as “The way to do research”.
   b. Systems medicine meeting in Berlin: Two presentations from INFECT were among the 10 presentations (out of 100 abstracts) selected for oral presentation.
2. A book volume will be published by Springer Nature publishing group (INFECT logo on the cover). The contract has been completed and the tentative publishing date is December 2019. The book will focus on NSTI and will target health care professionals and researchers/clinicians within the fields of infectious diseases, intensive care, microbiology and systems medicine and personalized medicine.
   a. A postgraduate training workshop for medical students and residents will be held in conjunction with the largest Nordic intensive care conference in 2019.
   b. A Youtube video currently being finalized, including clinicians, modellers and patients/relatives. This video is targeting the society at large.

Actions taken to the recommendations provided in the ethical review report

According to the ethical review report, an external ethics expert was assigned who has conducted annual reviews of all ethical aspects. These reports have been submitted with the periodic reports; all reports confirming a sound ethical approach in INFECT.

Potential Impact:

As outlined in annex I, INFECT had several expected impacts specified linked to the specific areas listed below. Herein, we report the actual impact achieved for respective area. The different colors in the tables (appendix) indicate the estimated time line; green = done/short-term (within 2018); blue = mid-term (within 2 years); red = long-term (> 2 years).

• Novel insights into the pathophysiology of NSTIs (Table 1; appendix)

*See section 4.1.3 for details of the results/achievements

The integrated systems biology approach used in INFECT identified several host and pathogen traits/pathways that contributed
to the severity and outcome of NSTIs. The findings provide critical data to support personalized medicine approaches in infectious diseases, and identifies targets for diagnostics, patient stratification and intervention. Some of which are already being pursued by the consortium in planned future studies (these are highlighted in green). Also some of the findings have already resulted in a changed clinical practice, such as in the case of biofilm revealing the need to change type of antibiotics to achieve an efficient bacterial clearance. The knowledge acquired is not only useful in the field of NSTIs, but will also be useful for other severe invasive infections, such as sepsis and its complication that is causing a significant health burden worldwide.

• Patient benefit: superior diagnosis (Table 2; appendix)

A key goal of INFECT was to promote improved diagnostics, as timely diagnosis is critical for these acute rapidly progressing infections. For this, we developed innovative diagnostic tools based on multiplex technology, and validation in the clinical setting was undertaken, which revealed potential of the new tool but also need for further optimization.

• Patient benefit: advanced understanding of the clinical aspects of NSTI and preparation of guidelines for management and care (Table 3; appendix).

A key achievement of INFECT was the enrollment of NSTI patients and the creation of the world’s largest patient cohort and associated biobank. Analyses of the comprehensive clinical registry generated an advanced understanding of these patients and provided documentation that has previously been lacking. This has been disseminated through scientific conferences and scientific publications, and importantly the process of creating evidence-based guidelines have been initiated through the involvement of proper clinical organisations (e.g. SSAI).

• Patient benefit: novel therapeutic strategies (Table 4; appendix)

INFECT has utilized the clinical registry containing treatment data, analyses of biobank samples, and even conducted a clinical trial (Madsen et al Int Care Med 2017). This has resulted in change of clinical practice and provided evidence that “one size” does not fit all patients. Further strengthening the importance of patient stratification and tailored therapy in infectious diseases. The results also have an impact on the use of antibiotics as it promotes the timely administration of the right type of antibiotic usage, which is of utmost importance in regards to antibiotic resistance development; a major global health threat.

• Design/optimization of future clinical trials (Table 5; appendix)

The novel insight on host and pathogen traits as detailed above shows the need for patient stratification and tailored therapy. One such host trait is currently being pursued by the consortium in planned future trial, and many more are in the pipeline. This is an important step towards achieving personalized medicine in NSTIs and improved patient outcome.

• Reduction on health care costs (Table 6; appendix)

In addition to the suffering of the patients and their relatives, the costs associated with these infections are substantial and represent a great burden to health care. IVIG as a biological is associated with substantial costs per patient, and hence, our finding that IVIG should not be used for all NSTI irrespective of aetiology but rather on a particular patient subpopulation is associated with direct health cost reductions. Also the results guiding antibiotic use is most valuable as timely and appropriate antibiotic use is critical in infection outcome.

• Establishment of the value of systems medicine in solving complex human diseases (Table 7; appendix)

Through the integrated systems biology approach in INFECT utilizing clinical data, different clinically relevant experimental
models and computational modeling, INFECT achieve an in-depth understanding of the pathophysiology of the multifactorial NSTIs and their co-morbidities; thereby identifying novel diagnostic and therapeutic targets. Importantly, the data demonstrated the need for patient stratification and tailored intervention and provided the insight necessary to create new concepts for this. Taken together, this shows the value of systems medicine in promoting medical advances in infectious diseases. The results of INFECT has demonstrated the impact of systems medicine to solve important health challenges.

• Fostering the competitiveness of SMEs and European innovation (Table 8; appendix)

One of the five objectives of the ambitious Lisbon 2020 agenda is to foster innovation and to starkly improve the competitiveness of the European industry, in particular SMEs. The long-term success of SMEs depends on the quality of services that they provided and the importance of these services to potential customers. Cube Dx, as the successor of Anagnostics GmbH, could further develop its highly multiplexed biomarker diagnostics and turn it into a potential point of care tool. The technology now provides a single step usage (filling in the sample) and allows whole blood as a sample. The technology has the potential to quantify more than 100 protein biomarker in about 13 minutes. The technology is therefore perfectly suitable to test whole biomarker profiles in clinical settings. Furthermore, Cube Dx could close the gap in its product range to identify pathogens on a molecular basis (DNA) and can now offer a highly effective and fast method to enrich pathogens from whole blood, beside the test to identify a wide range of relevant bacteria, resistance genes and fungi within less than 3 hours from whole blood. Both (platform) technologies boost Cube Dx’ competitiveness in the global diagnostics market. The major activities and commercial goals of the SMEs LifeGlimmer involved in INFECT include providing methods and workflows for the understanding of complex biological systems using systems medicine approaches. As envisaged, the developments in the application of modeling and bioinformatics approaches and tools herein developed have boosted the competitiveness of LifeGlimmer GmbH because they i) furthered product development and services of reverse engineering, dynamic, constrained-based and machine learning modeling towards the unraveling the mechanisms underlying disease and to predict potential biomarkers and intervention strategies, ii) significantly contributed to improve its product portfolio by creating new specialised tools; iii) enabled it to validate its main strategy in developing tools that can be used for analysis and interpretation of complex, high-volume data sets, and clinical data. This has been and will continue to be paramount to strengthen LifeGlimmer’s position as high-value service provider in the Systems Medicine and Health landscape.

• Training of early stage and experienced researchers (Table 9; appendix)

The INFECT project has included training of clinical and preclinical researchers, including preparation of educational material, training of PhD student as well as residents and medical staff. This training in an outstanding multi-disciplinary research environment has served to support the development of excellent research scientists in the fields of infectious diseases, intensive care, microbiology, and systems medicine. This training does not only offer highly trained research scientists to meet the employment demands of stakeholders in relevant R&D and commercial sectors, but also serves to foster the new generation of scientist in the new field of systems medicine in infectious diseases.

Dissemination activities

In INFECT a great emphasis has been on disseminating the knowledge generated in the project to increasing the awareness of the life-threatening NSTIs, inform about the above reported advances/knowledge created in INFECT, continuously working towards the translation of these results to improve patient care and outcome, as well as working towards the implementation of systems medicine in infectious disease and intensive care, thereby enabling personalized medicine approaches to be applied to also acute life-threatening infections.

The high quality of the work performed and the dissemination activities (as detailed in 4.1.3 and tables below) is evident by the following facts:
1. The high quality publications published in well renowned journals within the fields of infectious diseases, intensive care, microbiology and systems medicine
2. Invitations to leading meetings in infectious diseases, intensive care, microbiology and systems medicine both in Europe and outside (e.g. the Lancefield conferences in 2014 and 2017, European conference on Infectious diseases and clinical microbiology). At these and other meetings, INFECT has gained a lot of positive attention and its efforts acknowledged.

3. Invitation by a major publishing company to edit and produce a book volume on NSTI with a focus on the INFECT project.

4. Established collaborative efforts and support from the clinical scientific community (e.g SSAL) for training and guidelines preparations.

List of Websites:
Public website: www-fp7infect.eu

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**Related information**

[Documents and Publications](final1-appendix-1-figures-and-tables.pdf)

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